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Monoamino Monocarboxylic Acid Content of Preparations of Herbage Protein

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The similarity in amino acid composition of numerous 'representative' leaf proteins and of the constituent cytoplasmic and chloroplastic fractions is fairly well established but some doubt remains as to the degree of similarity, variations with age and species having been reported (review by Lugg, 1949).

Earlier analyses were rendered difficult by the dearth of suitable techniques and have, in general, been confined to those amino acids which are readily determined by means of some characteristic group in the molecule. Unfortunately, it is often these substances that are most labile under the conditions of hydrolysis.

Before investigating the fate of amino acids liberated by proteolysis in herbages under conditions of starvation (e.g. wilting and ensilage) it was essential to know whether the residual-protein fractions were of constant composition or whether it would be necessary to undertake analysis in every case. The method of Kemble & Macpherson (1954), which was developed primarily for the amino acid analysis of plant extracts and which was to be used for the proteolytic investigations, was therefore applied to the analysis of protein-rich fractions extracted by several methods from a variety of herbages.

EXPERIMENTAL

Source and extraction of proteins

Protein A. The protein was extracted from perennial rye grass (S. 24 hay strain) by the method of Crook (1946), which entails repeated grinding with sodium borate buffer at pH 8·0. The grass was senescent, being cut in December. 62·5% of its total protein-N was extracted, as material having N 10·8% of dry matter.

Protein B. Mixed grass from permanent pasture was macerated in 0·05M borax, filtered through paper pulp and precipitated at pH 4·5. The protein, after heat coagulation, was washed with ethanol and ether. 41·2% of the grass protein-N of the grass was obtained as material having N 14·5% of dry matter.

Protein C. Young lawn grass (height 4–6 in.) was macerated with a solution of borax (2%, w/v). After filtration through muslin the solution was brought to pH 4·5 and the protein coagulated by heating to 90°. 38·2% of the total protein-N was obtained; N, 8·9% of dry matter.

Protein D. Young perennial rye grass (S. 24 hay strain) was cut in May (height 4–6 in.). It was finely minced and the sap filtered by suction through muslin and then through paper. The protein was coagulated by heating to 90° at the pH of the sap (6·3). It was washed with hot water, ethanol and ether and represented 32·8% of the fresh grass protein-N; N, 13·4% of dry matter.

Protein E. A sample of alsike clover was cut before flowering and extracted as described for protein *A*. 39% of the clover protein-N was obtained as material having N 14·3% of dry matter.

Protein F. A similar grass sample to that used for protein *C* was allowed to wilt in the laboratory for 48 hr. In this time 10% of the protein had been degraded. The remainder was extracted as described for protein *C*. 37% of the total protein-N was extracted; N, 9·0% of dry matter.

Protein G. An identical sample of sap to that from which protein *D* was obtained was bottled, covered with a thick layer of liquid paraffin and placed in an incubator at 30°. These conditions simulated the ensilage process (Macpherson, 1952) and the pH dropped rapidly to 3·9. After

Table 1. *Monoamino monocarboxylic acid content* of seven herbage proteins*

Preparation	N as % total N							
	A	*B*	*C*	*D*	*E*	*F*	*G*	Barley (Yemm & Folkes, 1953)
Glycine	6·8	6·4	6·9	6·7	7·0	6·9	6·7	6·7–6·9
Serine†	4·2	4·1	4·2	4·0	3·9	4·0	3·6	3·3–3·5§
Threonine†	4·3	4·3	4·4	4·5	4·2	4·2	3·9	4·0§
Hydroxyproline					Absent			
Alanine	5·9	5·7	5·8	5·9	5·9	5·8	6·0	7·9–8·3
Proline	3·5	3·6	3·7	3·6	3·8	3·6	3·4	3·9–4·1
Tyrosine‡	1·7	1·7	1·5	1·7	1·6	1·6	1·7	2·2–2·3
Valine	4·4	4·8	4·7	4·8	4·7	4·5	4·5	4·9–5·1
Phenylalanine	3·5	3·6	3·5	3·6	3·3	3·5	3·6	3·0–3·2
Isoleucine	3·4	3·3	3·4	3·4	3·5	3·4	3·5	4·2–4·7
Leucine	5·7	5·3	5·9	5·5	5·7	5·3	5·4	5·8–6·0
Insoluble humin	4·2	1·6	3·2	4·9	1·1	6·1	3·9	2·4–4·3
Ammonia (formed on hydrolysis)	8·7	8·8	8·8	8·9	7·5	9·4	8·8	5·8–6·0

* Individual methionine values (0·2–0·8) are not recorded due to known heavy losses by this method of hydrolysis (Bailey, 1937).

† Corrected for hydrolysis losses (Rees, 1946).

‡ Minimum as from acid hydrolysate.

§ Uncorrected for hydrolysis losses.

15 weeks a precipitate (coagulated protein) was removed by filtration. Determination of the soluble nitrogen showed that 33% of the original protein had been degraded. The precipitate was washed as described for protein *D*; N, 9·7% of dry matter.

Analysis

Hydrolysis. Samples (250–350 mg.) of protein were boiled under reflux for 24 hr. with 500 ml. 6 N-HCl and taken to dryness *in vacuo*. The water-soluble material was separated from the insoluble humin by filtration and used for analysis.

Nitrogen determinations. These determinations were carried out by the micro-Kjeldahl technique (Chibnall, Rees & Williams, 1943).

Amino acid determinations. Analysis was carried out by the method of Kemble & Macpherson (1954).

RESULTS AND DISCUSSION

The proteins examined were obtained from individual species of different plant families (proteins *A*, *D* and *E*) and from uncontrolled mixtures of grasses (proteins *B* and *C*). The parent plants differed in stage of growth and the proteins varied greatly in purity and method of extraction. The latter variation implies a difference in the cytoplasmic/chloroplastic ratio, this being relatively high for *D* and low for *A*.

In spite of these variables, no significant difference could be detected in the monoamino monocarboxylic acid content of the proteins (Table 1). Furthermore, the results, other than for isoleucine and alanine, are in good agreement with those obtained by microbiological analyses of barley proteins (Yemm & Folkes, 1953).

The humin figures are not high considering the impurity of some of the preparations and this is probably due to the great dilution of the protein during hydrolysis (HCl: protein = 300:1, w/w). Even so, much destruction of methionine and probably some destruction of tyrosine has occurred.

In view of these results, it would seem reasonable to assume a consistent monoamino monocarboxylic acid composition for representative herbage proteins. However, when following the proteolysis which is a normal accompaniment of plant starvation, knowledge of the original protein composition is of no value unless it is also known if its breakdown is uniform. Hence two additional protein preparations (*F* and *G*) were analysed after extraction from grasses undergoing different types of starvation (wilting and ensilage respectively). Protein *F* gave an analysis typical of the fresh herbage proteins but protein *G* showed small but measurable diminutions in the values for the hydroxyamino acids, serine and threonine (Table 1).

This may imply some preferential release of these two amino acids. However, the protein had remained in contact with a solution of pH 3·9 for 15 weeks and since the hydroxyamino acids are the most labile under acid conditions, the possibility of some purely chemical degradation cannot be excluded. Apart from these two slight discrepancies the analysis was typical of a fresh herbage protein.

These findings demonstrate that protein breakdown during starvation is uniform in that the amino acid composition of the residual protein is virtually the same as that of the original.

SUMMARY

1. Application of a new and accurate method of analysis has not shown any significant difference in the monoamino monocarboxylic acid composition of representative herbage proteins.

2. Analysis of a protein extracted from wilted grass was not significantly different from that of fresh grass.

3. Analysis of a protein extracted from ensiled grass showed significant differences only in its content of serine and threonine, and these differences were small.

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Liberation of Amino Acids in Perennial Rye Grass During Wilting

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Proteolysis during starvation of plant material gives rise to an increase in peptide, free amino acids and amides. The amides appear in amount greater than that contained in the protein and must incorporate nitrogen liberated by deamination of amino acids. Thus some of the amino acids released by proteolysis are metabolized further and do not appear in the calculated amount. For example, Wood & Cruikshank (1944) have shown cystine, glutamic acid, arginine, tyrosine and tryptophan to be oxidized in that order of rapidity.

No evidence is available concerning the remainder of the amino acids and the investigation has been extended to include the majority of the monoamino monocarboxylic acids.

EXPERIMENTAL

Analysis

Total nitrogen. The total nitrogen (TN) is that nitrogen in the plant determinable by the micro-Kjeldahl technique (Chibnall, Rees & Williams, 1943). All results are expressed as a percentage of this TN.

Soluble nitrogen (SN). This is the fraction of the TN that is soluble in boiling water. Three successive extractions of 250 g. fresh material were made, each by 1 l. of boiling water.

Volatile base. The volatile base was determined by distilling a portion of the SN extract at pH 10·5 into standard acid.

Total amide. Asparagine and glutamine were hydrolysed by making a sample of the SN extract N with respect to H_2SO_4 and boiling under reflux for 3 hr. The increase in volatile base measures the NH_3 formed (Vickery, Pucher, Clark, Chibnall & Westall, 1935).

Glutamine. A sample of the SN extract was boiled under reflux at pH 6·5 for 2 hr. (Vickery *et al.* 1935). The increase in volatile base measures the NH_3 formed by the hydrolysis of glutamine. Some hydrolysis must have occurred during extraction but the resulting error cannot be great.

α-Carboxyl nitrogen. This was determined on a sample of the SN extract by the ninhydrin–CO_2 titrimetric procedure of Van Slyke, MacFadyen & Hamilton (1941).

Peptide. A sample of the SN extract (approximately 1 mg. N/ml.) was treated with 3 parts by vol. of ethanol. The resulting precipitate was removed by centrifuging and the N content of the precipitate determined. It is not claimed that this precipitate necessarily contains all the peptide present, but at least no α-carboxyl nitrogen is precipitated under these conditions and the determination is of value for comparison purposes.

Monoamino monocarboxylic acids. The ethanol was distilled from the supernatant solution obtained during the previous determination and a sample containing about 30 mg. N was subjected to ionophoresis. The neutral amino acids were estimated by the method of Kemble & Macpherson (1954*a*).

First wilting experiment

Young perennial rye grass (S. 24 hay strain; moisture content 88·3%; N, 4·56% of dry matter) was cut on 17 June 1953. The grass was 6–8 in. in height.

Six 250 g. samples were taken and the first was extracted immediately with boiling water. The remaining five samples were spread thinly on a laboratory bench and the SN was extracted after 1, 2, 3, 5 and 8 days respectively. The air temperature during the experiment was 14–18°.

Second wilting experiment

A mature sample of perennial rye grass (moisture content, 81·0%; N, 2·07% of dry matter) was cut on 28 October 1953. The grass (12–18 in.) was an aftermath and contained no flower heads.

Two samples (69·6 g. each) were taken. The first was allowed to wilt as before. The second was placed in a tall bottle and a slow stream of air saturated with water vapour was passed through the bottle continuously.

The SN was extracted from each after 3 days.